Review Article

# Considerations for Group Testing: A Practical Approach for the Clinical Laboratory

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**Abstract**
Group testing, also known as pooled sample testing, was first proposed by Robert Dorfman in 1943. While sample pooling has been widely practiced in blood-banking, it is traditionally seen as anathema for clinical laboratories. However, the ongoing COVID-19 pandemic has re-ignited interest for group testing among clinical laboratories to mitigate supply shortages. We propose five criteria to assess the suitability of an analyte for pooled sample testing in general and outline a practical approach that a clinical laboratory may use to implement pooled testing for SARS-CoV-2 PCR testing. The five criteria we propose are: (1) the analyte concentrations in the diseased persons should be at least one order of magnitude (10 times) higher than in healthy persons; (2) sample dilution should not overly reduce clinical sensitivity; (3) the current prevalence must be sufficiently low for the number of samples pooled for the specific protocol; (4) there is no requirement for a fast turnaround time; and (5) there is an imperative need for resource rationing to maximise public health outcomes. The five key steps we suggest for a successful implementation are: (1) determination of when pooling takes place (pre-pre analytical, pre-analytical, analytical); (2) validation of the pooling protocol; (3) ensuring an adequate infrastructure and archival system; (4) configuration of the laboratory information system; and (5) staff training. While pool testing is not a panacea to overcome reagent shortage, it may allow broader access to testing but at the cost of reduction in sensitivity and increased turnaround time.

## Background

Coronavirus disease 2019 (COVID-19) was declared a pandemic by the World Health Organization (WHO) on 11 March 2020.[1] Since then, it has induced lockdowns of varying severity in many countries. At the forefront of the pandemic, clinical laboratories are under immense pressure to escalate testing capacity despite facing a global supply shortage of reagents.[2] Group testing is seen as one of the solutions to mitigate supply shortages.[3] In this article, we review some of the criteria used to assess the suitability of an analyte for pooled sample testing and highlight a practical approach that a clinical laboratory may undertake to implement pooled testing.

Group testing, also known as pooled sample testing, was first proposed by Professor Robert Dorfman in 1943 at the height of World War II.[4] At that time, the Wassermann complement fixation test and Kahn flocculation test were used for the diagnosis of syphilis.[5,6] To provide sufficient reagents to screen all potential American enlistees, Dorfman proposed pooling multiple samples. If a pool is positive, then its constituent samples are analysed individually. If a pool is negative, all constituent samples are regarded as negative. In low disease prevalence, the Dorfman pooling strategy saves reagents.[7]

We propose five criteria to assess the suitability of an analyte for pooled sample testing:

1. The analyte concentrations in the diseased persons should be at least in the order of one magnitude higher than in healthy persons.
2. Sample dilution should not reduce the clinical sensitivity excessively.
3. The current prevalence of the disease must be sufficiently low for the number of samples per pool for the specific protocol.
4. There is no requirement for a fast turnaround time.
5. There is an imperative need for resource rationing to maximise public health outcomes.

**Criterion 1: Analyte concentrations in diseased persons should be at least one order of magnitude higher than in healthy persons**

For group testing to work, the distribution of analyte concentrations in diseased persons should be consistently higher than the analyte concentration in healthy persons, ideally with at least a difference of one order of magnitude (10 times). This enables the diluted pool concentration to be significantly higher than the upper limit of the healthy reference interval.

Consider a distribution (**Figure 1a**) of healthy and diseased individuals where the disease prevalence is 30% and the distributions overlap slightly. If a pool size of 2 is used, the possible outcomes are pools that contain (**Figure 1b**):

- two healthy samples
- two diseased samples
- one healthy and one diseased sample.

The pool that contains a mixture of samples from one healthy and one diseased individual will overlap with both the pool that contains samples from 2 healthy individuals and the pool that contains samples from 2 diseased individuals. This analyte distribution among diseased and healthy persons is not suitable for group testing.

Conversely, for an analyte where the distribution of diseased individuals is much higher than healthy individuals (**Figure 2a**), the distribution of the pools will not overlap. Even when the pool comprises of samples from 1 healthy and 1 diseased, the pooled concentration is sufficiently far from the 'cut-off of healthy individuals' or upper reference limit (**Figure 2b**).

Among chemistry analytes, few satisfy the criteria of diseased concentrations greater than healthy concentrations by at least one order of magnitude. Exceptions may include tumour markers and pituitary hormones. In multiple myeloma, free kappa and free lambda serum concentrations may exceed reference intervals by several orders of magnitude.[8,9] However, for a tumour marker to be several orders of magnitude higher than the upper reference limit, patients will

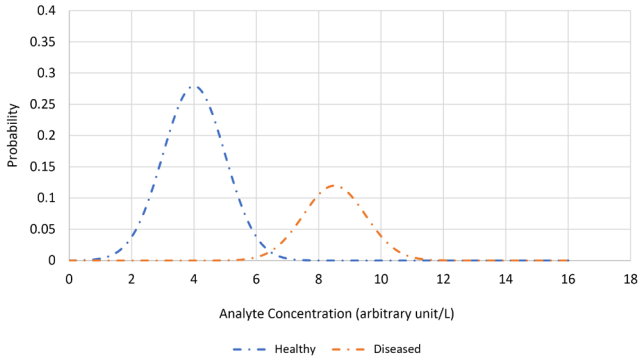


**Figure 1a.** Analyte concentration in healthy and diseased individuals for a typical chemistry analyte.

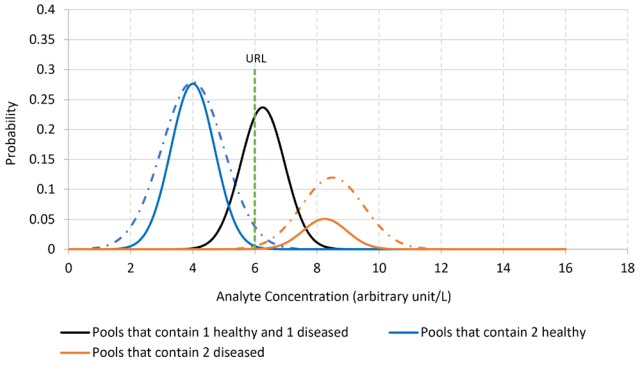


**Figure 1b.** Analyte concentration in healthy and diseased individuals for a typical chemistry analyte after pooling in groups of two. Solid lines represent the resultant pools. URL is the upper reference limit. Notice the overlap in concentrations after pooling which makes the typical analyte unsuitable for group testing.

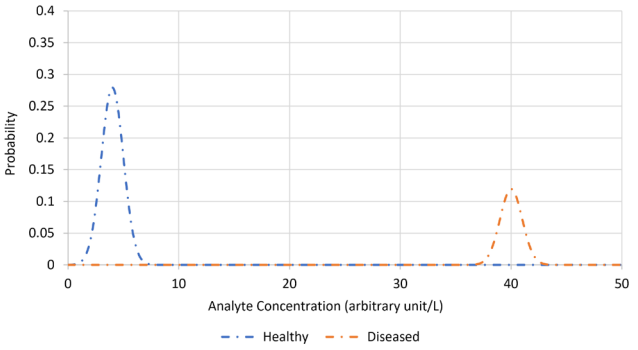


**Figure 2a.** Analyte concentration in healthy and diseased individuals for a hypothetical analyte where difference between the two groups is large (at least one order of magnitude).

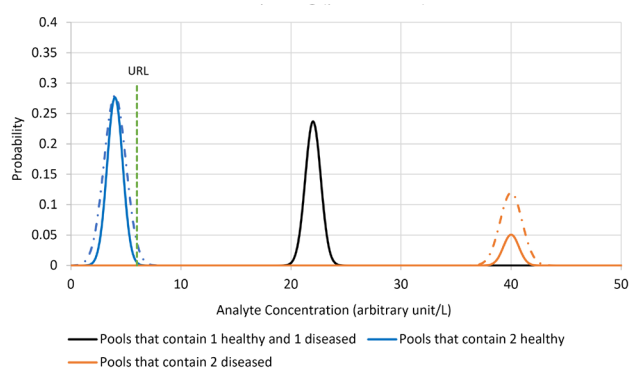


**Figure 2b.** Analyte concentration in healthy and diseased individuals for a hypothetical analyte after pooling in group of two. Notice the distribution of the pool that contains one diseased and one healthy sample (black solid line) does not overlap with the upper reference limit (URL).

often be at an advanced stage of disease. Pituitary hormones, particularly thyrotropin (TSH), in diseased individuals may exceed the upper reference limit by one order of magnitude, due to its inverse log-linear relationship with serum free thyroxine.[10-12] Historically, group testing had been used for TSH measurements during the infancy of immunochemistry where it was difficult to get stable and consistent reagents (Kallner A, personal communication).

In the context of infectious diseases, viral load is present in infected patients and absent in non-infected persons. The theoretical magnitude difference between the two categories is infinite, with no overlapping distributions. Infectious diseases have therefore been favourable candidates for group testing. Nucleic acid amplification in mini-pools for hepatitis B virus (HBV),[13,14] hepatitis C virus,[15] human immunodeficiency virus[16] and West Nile virus[17] for blood banking are widely practiced.[18] Screening of chlamydia and gonorrhoea using pooled samples has also been performed.[19,20] The pooled sample concentration (viral load) should be above the limit of detection (LoD), which is analogous to the cut-off for healthy individuals in a pooled chemistry test. If the viral load distribution is several orders of magnitude above the LoD, group testing is ideal. In blood banking, mini-pools of 512 and pools of up to 1200 have been used, with a diluted viral load still above the LoD (**Figure 3**).[21]

**Criterion 2: Sample dilution should not reduce clinical sensitivity excessively**

***Reduced Sensitivity from Sample Dilution***

Diluting a positive serum from an infected patient with negative sera from healthy persons will inevitably reduce analyte concentration and hence reduce detectability, ceteris paribus. Recently, the trend in blood banking is to use individual-donor nucleic acid amplification test or smaller pools of 4 to 16 because of sensitivity concerns.[22-24] Boland *et al.* showed that 59% of donors with a low viral load of 450 IU/mL for hepatitis E virus might have screened negative in a mini-pool of 24.[25] For Zika virus screening in blood banking, mini-pools created by 1 in 6 dilution using the investigational nucleic acid amplification test (NAT) only detected 251 of the 356 (71%) confirmed positive donations.[26] For HBV samples with viral load below 20 IU/mL, Chatterjee *et al.* showed that dilutions of 1:6 or 1:8 resulted in the detection of only 9 out of 27 replicates (33.33%).[27]

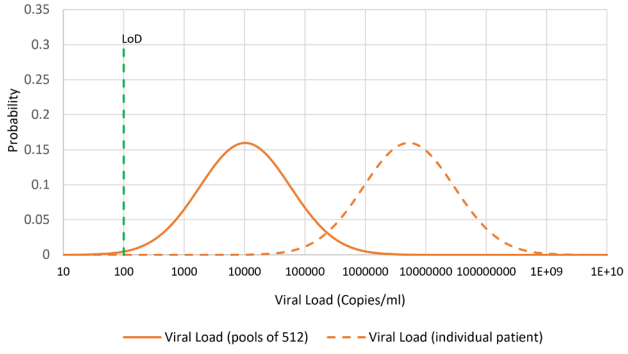


**Figure 3.** Pictorial representation of the viral loads among individual tested sample and pools of 512. The analyte is suitable for pooled testing, provided that the viral load in the pooled sample is still above limit of detection (LoD) of the assay.

For viral ribonucleic acid (RNA) reverse transcriptase (RT) polymerase chain reaction (PCR), a ten-fold dilution corresponds to a delay in Cycle Threshold (CT) value of $\log_2 10 = 3.32$ assuming a 100% PCR efficiency. For example, a weakly positive sample with a cycle threshold of CT 32 performed by an assay with a positivity cut-off of CT 35, will have a CT value of 35.32 if diluted ten-fold, entering the inconclusive/indeterminate zone.

There is an imprecision associated with each PCR CT value due to variability including fluorescence measurements, PCR efficiencies of the polymerase/primer/template complex, pipetting transfer volume and temperature variations.[28-30]

We may borrow concepts used in sigma metric and total allowable error (TEa) to highlight the impact of dilution. Consider the case where a laboratory is seeing weakly positive samples of CT value 32 and would like to embark on a pooling exercise. The pool size chosen is 4, hence the expected delay in CT is 2 ($\log_2 4 = 2$) given 100% efficiency. We assume the imprecision of the method is approximately CT 0.5, CT values are approximately normally distributed, the positivity cut off for the existing method is 35 (**Figure 4**) and no adjustment is made to the CT cut-off. We have taken the liberty to work with CT values directly as it is the most

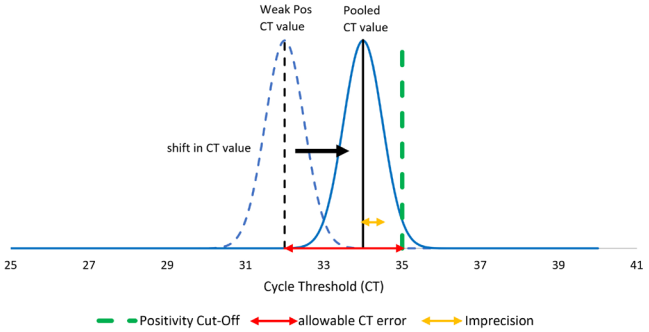


**Figure 4.** Shift in Cycle Threshold (CT) value of a weakly positive sample after pooling into groups of 4 with consequent reduction in sigma metric. Assay imprecision = 0.5, positivity cut-off = 35. The expected CT can be calculated by $\log_2$(number of samples pooled). Consequently, the CT value of this positive pool shifted closer towards the limit of detection (LoD).

common unit of measurement in a routine clinical laboratory for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RT-PCR testing. A formal coefficient of variation and standard deviation should be recalculated from specific units (copies/mL or u/L) instead of CT values.[32] Imprecision may not be normally distributed in practice. At a very low number, the actual number of viral copies present in a reaction cell follows Poisson distribution.[33,34]

Given that,

- Sigma metric[31] = $\frac{TEa - Bias}{Imprecision}$

Similarly,

- Sigma metric for weakly positive NAT samples after pooling

$$= \frac{Allowable\ CT\ error\ -\ expected\ CT\ change}{Imprecision\ of\ CT}$$

$$= \frac{(\text{positivity cut off CT} - \text{CT value of weakly positive sample}) - log_2(\text{number of pooled sample})}{Imprecision\ of\ CT}$$

In this example by pooling 4 samples, the sigma metric for a low CT sample has decreased from $\frac{(35-32)}{0.5} = 6$ to $\frac{(35-32)-2}{0.5} = 2$.

The loss in sensitivity can also be illustrated using a probit plot. Consider a probit plot for a PCR assay (**Figure 5**), with a hit rate of 95% at 14 copies per reaction. For an eluate with 40 copies per reaction, expected hit rate is 100% (yellow filled circle). After four-fold dilution, expected hit rate decreases to approximately 88% (red filled circle) in this example.

### *Ways to Overcome Reduced Sensitivity*

To enable equivalence post dilution compared with the existing method, a more sensitive assay with a lower LoD should be used. For example, instead of using an assay where the LoD is 100 copies/reaction, we may use an assay with LoD of 10 copies/reaction if the pooling size is 10, as the sensitivity of pools is dependent on the original sensitivity of the method.[35]

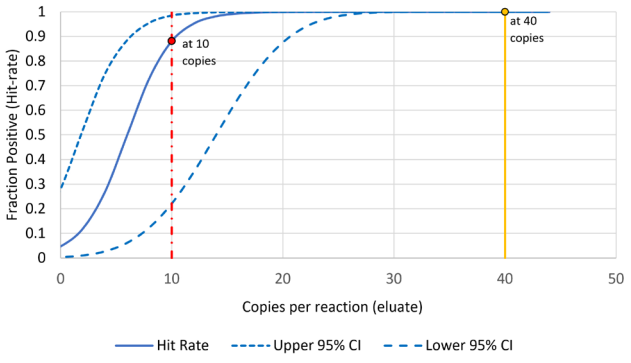


**Figure 5.** A Probit Plot of Fraction Positive (Hit-Rate) at various concentrations of viral copies (blue solid line) for a Polymerase Chain Reaction (PCR) assay. Dashed blue lines are the associated confidence intervals. This plot illustrates the reduction in hit rate due to sample dilution. A low positive sample with eluate of 40 copies per reaction well (yellow filled circle) will have a hit rate of close to one. Dilution by 4x will reduce the number of copies per reaction well from 40 to 10 (red-filled circle), reducing the hit-rate.

Another option is to use the same assay, but to analyse the constituent samples of inconclusive/indeterminate pools (e.g. CT threshold of 35–40) individually, so as to allow the detection of pooled samples that fall slightly below the LoD. The Interim Guidance for Use of Pooling Procedures by the Centers for Disease Control and Prevention (CDC) recommends that if a pooled result is indeterminate, then all the specimens in the pool need to be retested individually.[36]

Pooling a small number of samples should not adversely affect sensitivity for SARS-CoV-2 RNA PCR.[37] While Yelin *et al.* showed that pools of 32 may give a false negative rate of 10%, pools of 16 are able to achieve a sensitivity of 96%.[38] Other authors have implemented pool testing of n≤10 and found that pooling does not affect clinical sensitivity. By using a pool size of 10, Sahajpal *et al.* were able to identify 6 out of 6 positive samples among 940 de-identified samples that were previously tested for SARS-CoV-2, demonstrating proof of concept for mass population screening.[39] Ben-Ami *et al.* were able to detect 15 out of 15 pools with positive samples by using a pool size of 8 and successfully implemented their pooling protocol for their population.[40] Abdalhamid *et al.* used a pool size of 5 and were able to detect all 25 pools that contain positive samples on a CDC RT-PCR assay.[41] Expectedly, in the study by Abdalhamid *et al.* the mean delay in nucleocapsid gene N1 and nucleocapsid gene N2 CT cycles for the 25 pools was 2.67 and 2.24 respectively. This is close to the theoretical delay in CT of 2.32 cycles caused by a five-fold dilution ($\log_2 5 = 2.32$).

However, it is important to recognise that the false negative rate may vary with respect to the concentration of the original individual samples. If strong positives are pooled, the concentration of the diluted pools will be above the LoD. If weak positives are pooled, the resultant concentration of the diluted samples may be below the LoD. Laboratories may review their historical patient samples CT values and determine the percentage of their samples that are weakly positive which will fall below the LoD after dilution.

The aim is to provide a test of equivalent clinical sensitivity despite dilution of samples. In other words, the sensitivity penalty from dilution of samples must be met by sensitivity headroom. The sensitivity penalty can be reduced by pooling only a small number of samples. The sensitivity headroom

can be widened by using an existing method that is highly sensitive, implementation of a more sensitive assay, or proof that historical samples received by the laboratories are mainly in the moderate to high viral load that will be less affected by dilution.

**Criterion 3: Current prevalence of the disease must be sufficiently low for the pooling protocol used**

The crux of reagent savings lies in optimising the sample pool size with respect to the prevalence. It is important to understand the mathematics before embarking on a group testing protocol, as close monitoring of the prevalence is required. Thus far most pooled testing protocols implemented are based on the Dorfman protocol.

The Dorfman protocol involves pooling k samples per group and testing the group. If the group is negative, constituent samples are considered negative. If the group is positive, all the constituent samples are individually tested. This is the simplest and most practical approach for routine use.

We now discuss how the prevalence and pool size affect the number of tests used:

- Let N be the number of individuals for screening.
- Let p be the probability of selecting an individual that is infected (prevalence).
- Hence, probability of selecting an individual that is non-infected = 1 – p.
- Let k be the number samples per pool (pool size).
- Let G be the number of pools needing to be screened, where G
  = number of individuals for screening / number of samples per pool
  = N/k.
- Let θ be the probability that a single pool (that consists of k randomly selected samples) has at least one infected sample, hence θ
  = 1 – Prob(all samples in the pool are not infected)
  = $1 - (1-p)^k$
- Let X be the number of infected pools, among the total number of pools, G, screened.
  X follows binomial distribution, X ~ Binom (G, θ).
  Expected positive pools, $E(X) = G*\theta = \frac{N}{k} * [1 - (1-p)^k]$
- Total tests used, T = Total number of pools + Expected positive pools * Pool size
  = G + E(X)*k
  = $\frac{N}{k} + \frac{N}{k} * [1 - (1-p)^k] * k$.
- Tests used per individual, t = Total tests used / Number of individuals
  = $\frac{T}{N}$
  = $\frac{\frac{N}{k} + \frac{N}{k} * [1 - (1-p)^k] * k}{N}$
  = $\frac{1}{k} + 1 - (1-p)^k$
- In conventional individual testing scenario, 1 person will consume 1 test.
- Hence, savings = 1 – t
  = $1 - [\frac{1}{k} + 1 - (1-p)^k]$
  = $(1-p)^k - \frac{1}{k}$
- Savings therefore can be calculated using a surprisingly simple formula $(1-p)^k - \frac{1}{k}$, and is contingent on optimising with pool size, k and prevalence, p.

The optimal pool size for a particular prevalence for the Dorfman Protocol is shown in **Table 1**. If the prevalence is 1%, an optimal pool size of 11 will save 80.4% of tests. However even if we select a pool size smaller than 11, for example using a pool size of 2, we will still save 48.0% of tests for a prevalence of 1%.

The Dorfman Protocol is described as a two-stage adaptive protocol. It is 'two-stage' as it involves two stages - firstly testing all the pools, followed by testing constituents of positive pools. It is 'adaptive' as only the positive pools are further acted on (adapts to the new information given by the first stage testing or depends on the outcome of a previous test).[42]

Multi-stage adaptive approaches have been described, but may be difficult to implement clinically.[43] A binary splitting strategy has been studied, where positive pools are further split into two equal pools repeatedly until the positive sample can be identified, while constituent samples in negative pools are considered negative.[44] Eberhardt *et al.* has also proposed a three-stage testing scheme with pool sizes of maximum 16 samples.[45] His group approach can test up to three and seven times as many individuals with the same number of test kits for prevalence rates of around 5% and 1%, respectively, compared to 1.8 times and five times in the Dorfman Protocol. These multi-stage approaches have potential to save more reagents but at a cost of increased turnaround time and complexity.

On the other hand, one-stage non-adaptive pooling protocols involve only a single stage to identify all positive samples. A matrix pooling strategy, where $n^2$ samples are ordered in an n x n matrix and each row and column are pooled and tested, has been described. Positive samples are identified by the intersection of the columns and rows whose pools are positive.[46] For SARS-CoV-2 PCR, Ben-Ami *et al.* has experimented with a 5 x 5 matrix to test 25 samples.[40] A 4x4 matrix to test 16 samples has also been implemented by a private laboratory in the US.[47] The requirement for low prevalence remains. If there is more than one positive sample

**Table 1.** Percentage of tests saved by using Dorfman Protocol.

| | Prevalence | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pool Size | 0.01% (10-4) | 0.1% (10-3) | 0.5% (5x10-3) | 1% (10-2) | 2% (2x10-2) | 3% (3x10-2) | 4% (4x10-2) | 5% (5x10-2) | 6% (6x10-2) | 7% (7x10-2) | 8% (8x10-2) | 9% (9x10-2) | 10% (10-1) | 11% (1.1x10-1) | 12% (1.2x10-1) | 13% (1.3x10-1) | 14% (1.4x10-1) | 15% (1.5x10-1) | 20% (2x10-1) | 25% (2.5x10-1) | 30% (3x10-1) |
| 2 | 49.98% | 49.8% | 49.0% | **48.0%** | 46.0% | 44.1% | 42.2% | 40.3% | 38.4% | 36.5% | 34.6% | 32.8% | 31.0% | 29.2% | 27.4% | 25.7% | 24.0% | 22.3% | 14.0% | 6.3% | -1.0% |
| 3 | 66.6% | 66.4% | 65.2% | 63.7% | 60.8% | 57.9% | 55.1% | 52.4% | 49.7% | 47.1% | 44.5% | 42.0% | 39.6% | 37.2% | 34.8% | 32.5% | 30.3% | 28.1% | 17.9% | 8.9% | 1.0% |
| 4 | 75.0% | 74.6% | 73.0% | 71.1% | 67.2% | 63.5% | 59.9% | 56.5% | 53.1% | 49.8% | 46.6% | 43.6% | 40.6% | 37.7% | 35.0% | 32.3% | 29.7% | 27.2% | 16.0% | 6.6% | -1.0% |
| 5 | 80.0% | 79.5% | 77.5% | 75.1% | 70.4% | 65.9% | 61.5% | 57.4% | 53.4% | **49.6%** | 45.9% | 42.4% | 39.0% | 35.8% | 32.8% | 29.8% | 27.0% | 24.4% | 12.8% | 3.7% | -3.2% |
| 6 | 83.3% | 82.7% | 80.4% | 77.5% | 71.9% | 66.6% | 61.6% | 56.8% | 52.3% | 48.0% | 44.0% | 40.1% | 36.5% | 33.0% | 29.8% | 26.7% | 23.8% | 21.0% | 9.5% | 1.1% | -4.9% |
| 7 | 85.6% | 85.0% | 82.3% | 78.9% | 72.5% | 66.5% | 60.9% | 55.5% | 50.6% | 45.9% | 41.5% | 37.4% | 33.5% | 29.9% | 26.6% | 23.4% | 20.5% | 17.8% | 6.7% | -0.9% | -6.1% |
| 8 | 87.4% | 86.7% | 83.6% | 79.8% | 72.6% | 65.9% | 59.6% | 53.8% | 48.5% | 43.5% | 38.8% | 34.5% | 30.5% | 26.9% | 23.5% | 20.3% | 17.4% | 14.7% | 4.3% | -2.5% | -6.7% |
| 9 | 88.8% | 88.0% | 84.5% | 80.2% | 72.3% | 64.9% | 58.1% | 51.9% | 46.2% | 40.9% | 36.1% | 31.7% | 27.6% | 23.9% | 20.5% | 17.4% | 14.6% | 12.1% | 2.3% | -3.6% | -7.1% |
| 10 | 89.9% | 89.0% | 85.1% | 80.4% | 71.7% | 63.7% | 56.5% | 49.9% | 43.9% | 38.4% | 33.4% | 28.9% | 24.9% | 21.2% | 17.9% | 14.8% | 12.1% | 9.7% | 0.7% | -4.4% | -7.2% |
| 11 | 90.8% | 89.8% | 85.5% | 80.4% | 71.0% | 62.4% | 54.7% | 47.8% | 41.5% | 35.9% | 30.9% | 26.3% | 22.3% | 18.7% | 15.4% | 12.5% | 9.9% | 7.6% | -0.5% | -4.9% | -7.1% |
| 12 | 91.5% | 90.5% | 85.8% | 80.3% | 70.1% | 61.1% | 52.9% | 45.7% | 39.3% | 33.5% | 28.4% | 23.9% | 19.9% | 16.4% | 13.2% | 10.5% | 8.0% | 5.9% | -1.5% | -5.2% | -6.9% |
| 13 | 92.2% | 91.0% | 86.0% | 80.1% | 69.2% | 59.6% | 51.1% | 43.6% | 37.0% | 31.2% | 26.1% | 21.7% | 17.7% | 14.3% | 11.3% | 8.7% | 6.4% | 4.4% | -2.2% | -5.3% | -6.7% |
| 14 | 92.7% | 91.5% | 86.1% | 79.7% | 68.2% | 58.1% | 49.3% | 41.6% | 34.9% | 29.1% | 24.0% | 19.6% | 15.7% | 12.4% | 9.6% | 7.1% | 5.0% | 3.1% | -2.7% | -5.4% | -6.5% |
| 15 | 93.2% | 91.8% | 86.1% | 79.3% | 67.2% | 56.7% | 47.5% | 39.7% | 32.9% | 27.0% | 22.0% | 17.6% | 13.9% | 10.7% | 8.0% | 5.7% | 3.7% | 2.1% | -3.1% | -5.3% | -6.2% |
| 16 | 93.6% | 92.2% | 86.0% | 78.9% | 66.1% | 55.2% | 45.8% | 37.8% | 30.9% | 25.1% | 20.1% | 15.9% | 12.3% | 9.2% | 6.7% | 4.5% | 2.7% | 1.2% | -3.4% | -5.2% | -5.9% |
| 17 | 93.9% | 92.4% | 85.9% | 78.4% | 65.0% | 53.7% | 44.1% | 35.9% | 29.0% | 23.2% | 18.3% | 14.2% | 10.8% | 7.9% | 5.5% | 3.5% | 1.8% | 0.4% | -3.6% | -5.1% | -5.6% |
| 18 | 94.3% | 92.7% | 85.8% | 77.9% | 64.0% | 52.2% | 42.4% | 34.2% | 27.3% | 21.5% | 16.7% | 12.8% | 9.5% | 6.7% | 4.5% | 2.6% | 1.1% | -0.2% | -3.8% | -5.0% | -5.4% |
| 19 | 94.5% | 92.9% | 85.7% | 77.4% | 62.9% | 50.8% | 40.8% | 32.5% | 25.6% | 19.9% | 15.2% | 11.4% | 8.2% | 5.7% | 3.6% | 1.8% | 0.4% | -0.7% | -3.8% | -4.8% | -5.1% |
| 20 | 94.8% | 93.0% | 85.5% | 76.8% | 61.8% | 49.4% | 39.2% | 30.8% | 24.0% | 18.4% | 13.9% | 10.2% | 7.2% | 4.7% | 2.8% | 1.2% | -0.1% | -1.1% | -3.8% | -4.7% | -4.9% |
| 25 | 95.8% | 93.5% | 84.2% | 73.8% | 56.3% | 42.7% | 32.0% | 23.7% | 17.3% | 12.3% | 8.4% | 5.5% | 3.2% | 1.4% | 0.1% | -0.9% | -1.7% | -2.3% | -3.6% | -3.9% | -4.0% |
| 30 | 96.4% | 93.7% | 82.7% | 70.6% | 51.2% | 36.8% | 26.1% | 18.1% | 12.3% | 8.0% | 4.9% | 2.6% | 0.9% | -0.3% | -1.2% | -1.8% | -2.2% | -2.6% | -3.2% | -3.3% | -3.3% |
| 40 | 97.1% | 93.6% | 79.3% | 64.4% | 42.1% | 27.1% | 17.0% | 10.4% | 5.9% | 3.0% | 1.1% | -0.2% | -1.0% | -1.6% | -1.9% | -2.1% | -2.3% | -2.3% | -2.5% | -2.5% | -2.5% |
| 50 | 97.5% | 93.1% | 75.8% | 58.5% | 34.4% | 19.8% | 11.0% | 5.7% | 2.5% | 0.7% | -0.5% | -1.1% | -1.5% | -1.7% | -1.8% | -1.9% | -1.9% | -2.0% | -2.0% | -2.0% | -2.0% |
| 60 | 97.7% | 92.5% | 72.4% | 53.0% | 28.1% | 14.4% | 7.0% | 2.9% | 0.8% | -0.4% | -1.0% | -1.3% | -1.5% | -1.6% | -1.6% | -1.6% | -1.7% | -1.7% | -1.7% | -1.7% | -1.7% |
| 70 | 97.9% | 91.8% | 69.0% | 48.1% | 22.9% | 10.4% | 4.3% | 1.3% | -0.1% | -0.8% | -1.1% | -1.3% | -1.4% | -1.4% | -1.4% | -1.4% | -1.4% | -1.4% | -1.4% | -1.4% | -1.4% |
| 80 | 98.0% | 91.1% | 65.7% | 43.5% | 18.6% | 7.5% | 2.6% | 0.4% | -0.5% | -0.9% | -1.1% | -1.2% | -1.2% | -1.2% | -1.2% | -1.2% | -1.2% | -1.2% | -1.2% | -1.2% | -1.2% |
| 90 | 98.0% | 90.3% | 62.6% | 39.4% | 15.1% | 5.3% | 1.4% | -0.1% | -0.7% | -1.0% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% | -1.1% |
| 100 | 98.0% | 89.5% | 59.6% | 35.6% | 12.3% | 3.8% | 0.7% | -0.4% | -0.8% | -0.9% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% | -1.0% |

How to use this table:

- Locate the current prevalence in the columns
- Find the desired pool size in the rows
- The intersection of columns and rows represents the savings.
  - e.g. prevalence of 1% and pool size of 2, will save 48.0% of reagents as compared to testing patients individually (bolded)
  - e.g. prevalence of 7% and pool size of 5, will save 49.6% of reagents as compared to testing patients individually (bolded)

Savings is given by $(1-p)^k - \frac{1}{k}$, where p is prevalence and k is pool size.

Negative value indicates that more tests are consumed instead. Notice at prevalence of 30%, most pool sizes gave negative values. Dorfman Protocol is not efficient at higher prevalence. The theoretical optimal pool size (maximal savings) for prevalence of 0.5% to 25% is highlighted in yellow, while the optimal pool for 0.01% and 0.1% are 101 and 32 respectively (not shown).

among the n x n matrix, the positive column and row pools may intersect at multiple points, resulting in false positives (**Figures 6a, b and c**). If the prevalence is exceeded, a second stage individual testing of positive samples may be done to identify the false positives which incurs more reagents and time. Other one-step combinatorial pooling strategies have been proposed, but similarly require a low prevalence.[48]

**Criterion 4: There is no requirement for fast turnaround time**

The Dorfman protocol involves performing analysis on the pooled sample first, followed by the individual constituents if the pool is positive. For negative samples that are pooled with a positive sample, results will be held back at the group testing stage. Multistage adaptive protocols further worsen the turnaround time. One-stage non-adaptive protocols may also require longer processing time due to complex pipetting steps.

A hospital clinical laboratory typically receives samples from the emergency department, inpatient wards, primary care and from the community. Pooling samples for emergency department and inpatients may be inappropriate if the additional stages or complex pipetting steps delay medical intervention, discharge and transfer to step down facilities or render staff unable to determine the level of personal protective equipment (e.g. powered air-purifying respirator) in the operating theatre required for COVID-19 positive patients.[49,50]

**Criterion 5: Imperative need for resource rationing to maximise public health outcomes**

There should be a clear need for resource rationing before pooling is undertaken. This could be shortage of testing reagents or budget/skilled manpower constraints in a resource poor setting. In the early phase of the pandemic there was a global shortage of viral extraction kits and a need for extraction kit conservation.[51,52] At a prevalence of 1%, even a pool size of two can save 48% of reagents, doubling the tests produced by using the Dorfman Protocol, with a theoretical delay of just one CT and minimal loss in sensitivity. This compares well with the alternative method of direct PCR (with or without heat lysis) described by Fomsgaard *et al*. Furthermore, direct PCR (with or without heat lysis) as a replacement for nucleic acid extraction performs poorly for other PCR assays due to PCR inhibition.[53]

Molecular diagnostics requires skilled manpower and specialised equipment, therefore large scale testing could be prohibitive in resource poor settings.[54,55] Pooling represents an attractive solution to test large segments of the population, enables laboratories to address skill set shortages, increases

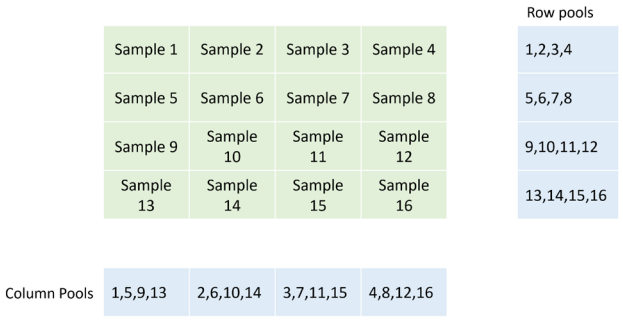


**Figure 6a.** Example of a matrix pooling (4 x 4). Step 1: Arrange 16 samples in 4 rows by 4 columns. Step 2: Pool the rows and columns. The first row pool comprises of samples 1, 2, 3 and 4. The first column pool comprises of samples 1, 5, 9 and 13.

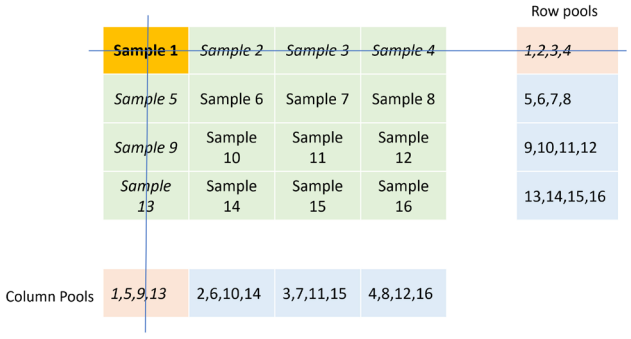


**Figure 6b.** One positive sample among the samples in the 4 x 4 matrix. If Sample 1 is positive, then first row and first column pools will be positive. By intersection of first row and first column, we can locate the Sample 1.

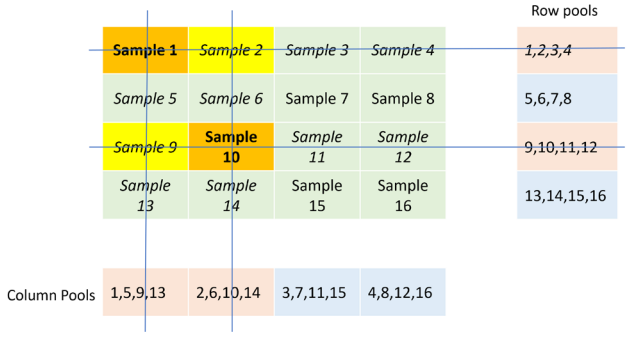


**Figure 6c.** Two positive samples among the samples in the 4 x 4 matrix. If Samples 1 and 10 are positive, then first row, third row, first column, second column pools are positive. By intersection of these rows and columns, we correctly identify Samples 1 and 10 but this also resulted in two false positives (Samples 2 and 9). Reagents savings is given by (n-2)/n. To always avoid multiple intersection points, prevalence should not be more than $1/n^2$. For a 4 x 4 matrix, savings are 50% and the prevalence should not exceed 6.25%. For a 5 x 5 matrix, savings are 60% and the prevalence should not exceed 4%.

density of production capacity and enables demand to be met with little increase in specialised equipment and space. Group testing using a pool size of ten contributed to Ghana's capacity to conduct over 370,000 SARS-CoV-2 tests between March and mid-July 2020, reducing the backlog of samples that had built up in the laboratories and relieving overcrowded isolation centres.[56,57]

In a review article, Walsh *et al.* noted there was little to no difference in SARS-Cov-2 viral load between pre-symptomatic, asymptomatic and symptomatic patients in seven studies.[58] Increased testing capacity may allow for positive case identification in the asymptomatic and pre-symptomatic population, who otherwise would not be screened. Pooled testing may reduce testing backlog.[59] When coupled with efficient contact tracing and physical distancing measures, reduction in backlog and minimisation of testing delay will decrease the Basic Reproduction Number and impact on pandemic control.[60]

However, the reduction in sensitivity from group testing presents a dilemma of increased false negatives, which must be mitigated as discussed in the previous section.

**A Practical Approach for the Clinical Laboratory**

With the five criteria satisfied, laboratories may proceed to implementation. We suggest five key steps for a successful implementation of SARS-CoV-2 PCR pooled testing when using the Dorfman Protocol:

1. Determining when pooling takes place (pre-pre analytical, pre-analytical or analytical stage)
2. Validating the pooling protocol
3. Ensuring adequate infrastructure and archival space
4. Configuration of the laboratory information system (LIS)
5. Staff training.

***Step 1: Determine When Pooling Takes Place***

It is important to recognise that pooling of samples may take place on at least three levels: pre-pre analytical, pre-analytical and analytical phases (not to be confused with the specific pooling protocol e.g. Dorfman, binary splitting discussed in Criterion 2).

Viral nucleic acid amplification testing involves collection and placement of the collected swab in transport media, arrival in laboratories, nucleic acid purification of media and amplification of the eluates. Hence, each stage presents an opportunity for pooling of samples (**Table 2**):

- Level 1 (pre-pre analytical): Multiple donor swabs may be pooled into a single media before transport to the clinical laboratory. Alternatively, for institutions that are using dry swabs, multiple donor dry swabs may be pooled into a single media (buffer/saline) on site.
- Level 2 (pre-analytical): Multiple donor transport media may be pooled in the laboratory before nucleic acid purification.
- Level 3 (analytical): Multiple donor purified nucleic acids (eluates) may be pooled the in laboratory before PCR.

In some countries, pooling may be done at the site of collection (Level 1).[61] The laboratory will therefore receive a pooled sample (with several swabs in a single transport media). The advantage of this approach is that the laboratory will regard the pooled sample as an individual sample in terms of handling, and the workflow remains the same. This strategy also saves transport media. There is also no dilution of the transport media and theoretically no reduction in sensitivity. The disadvantage of this strategy is if the pool is positive, swabs must be recollected from all the persons in the pool.[62] This strategy has the longest turnaround time and likely the highest manpower cost. Pooling at this stage can only be operationalised on a population where re-harvesting is guaranteed.

Level 2 pooling (multiple donor transport media are pooled before nucleic acid purification) is the most common approach, with savings in viral nucleic acid purification kits and PCR reagents. It increases turnaround time moderately, due to the need for re-extraction and re-amplification if the pool is positive.

Level 3 pooling (multiple donor eluates pooled before PCR) has also been performed, with savings in PCR reagents but not nucleic acid purification kits.[63] This strategy has the cleanest audit trail, lowest complexity and fastest TAT for retest and positive identification. This is as close to individualised testing in terms of consumption (storage, consumables, manpower, saturation of systems). Drawbacks include instability of RNA eluate.

Level 1 pooling requires the cooperation of external agencies and is difficult for a laboratory to implement alone. Level 2 and 3 pooling are most relevant to a clinical laboratory setting.

***Step 2: In-house Validation Protocol for Laboratory***

We suggest using the Dorfman protocol. The exact pool size (k) per group will be dependent on local prevalence according to Table 1 and limitations of sensitivity. The US Food and Drug Administration has provided a template for molecular laboratories which intend to pursue Emergency Use Authorisation for pooling of samples.[64] When adding a pooling strategy to a previously authorised test, some of the recommendations include:

**Table 2.** Summary of stages where pooling may take place.

| Level | Stage of Total Testing Process | Description | Location | Workflow | Turnaround Time* | Sensitivity | Reagent Savings | Comments |
|---|---|---|---|---|---|---|---|---|
| 1 | Pre-Pre Analytical | Pooling into single media (UTM/ VTM) at collection | Onsite | No Change | ↑↑↑↑ | = | Transport Media, extraction kits, RT-PCR master mix reagents | May only be operationalised if re-harvesting is guaranteed |
| 2 | Pre-Analytical | Pooling before nucleic acid extraction | In Lab | Require Adjustment | ↑↑ | ↓ | Extraction kits, RT-PCR master mix reagents | Most common |
| 3 | Analytical | Pooling before RT-PCR | In Lab | Require Adjustment | ↑ | ↓ | RT-PCR master mix reagents only | Clear audit trail |

*Turnaround time considerations - Level 1 pooling requires re-collection of samples from the individual patients if a positive pool is encountered. The need for re-collection of sample, transport, re-extraction and re-amplification results in the highest turnaround time. Level 2 pooling requires re-extraction and re-amplification. Level 3 pooling requires only re-amplification.

- Conducting a clinical validation study with a minimum of 20 individual positive samples collected from the intended use population using the laboratory's existing assay. Archived samples may be used if they have sufficient volume.
- Ensuring that at least 25% of the positive validation samples should be within 2-3 CT of the cut off (e.g. weak positive).
- Pooling positive samples with k-1 (e.g. where k = 5, k-1 = 4) randomly selected negative samples. The resulting pools should be tested by an existing assay.
- A plot of CT values for the sample pools on the Y axis and CT values for the individually tested samples on the X axis may be drawn. Perform regression analysis with slope and intercept along with 95% confidence interval. Using regression analysis, evaluate the shift in CT values for the positive patient samples diluted with negative patient samples.
- Ensuring that the clinical validation study should demonstrate that individual positive samples with viral load close to the assay's LoD (i.e. weak positives) are accurately detected in a pool of negative samples.
- Confirming that samples with negative results remain negative in k-sample pools. Testing 20 pools each consisting of k (e.g. k = 5) negative samples is recommended.

To validate a pooling protocol on an existing authorised assay, 20 positive pools may be tested. Each pool should contain one positive sample with the remainder negative samples. The desired pool size is determined by prevalence. For sensitivity analysis, comparison of CT values between the individually tested positive sample and positive pooled samples must be performed to assess the delay in CT value. A plot of CT values for the sample pools on the Y axis and CT values for the individually tested samples on the X axis may be drawn and compared by linear regression. For specificity analysis, 20 pools of negative samples for the same desired pool size should be performed.

***Step 3: Configuration of the Laboratory Information System***

An ideal LIS should be configured to support the laboratory workflow.[65,66] For a small sample pool (n≤3), two to three small patient labels (stickers) may be used to label a pooled microtube, with corresponding handwritten worksheets that link the primary accession numbers to the pooled sample identification number for traceability. For a large pool (n≥4), we recommend generating a secondary accession number in LIS. This secondary accession number should be registered to the individual accession numbers of the constituents. This may be done at sample reception. After accessioning, each pool with its constituent samples and secondary accession label may be placed in a basket for easy recognition, before

transportation to the molecular section.

Alternatively, the secondary accessioning can be done by the molecular staff, who will scan the individual constituent samples and assign a secondary accession number in the molecular section.

*Release of Results*
When the pool is tested negative, staff should only need to validate the negative secondary accession and the primary accessions associated with the individual samples will be automatically released with a comment that pooling was performed and the pool was found to be negative.

***Step 4: Ensuring Adequate Infrastructure and Archival Space***
Additional bench space and biosafety cabinets are required to temporarily accommodate the primary sample tubes when the secondary (pooled) tubes are undergoing testing. If pooling takes place at the analytical level (Level 3 – after extraction and before PCR), additional cold blocks/freezers are needed to house the eluate.

***Step 5: Training of Staff***
Laboratory staff should be familiar with the concepts, workflow and pitfalls of pooling. Pooling involves more steps and risks misidentification. Laboratory staff must cope with distinctly different workflows between individual inpatient samples and pooled community samples. Work involving large pool sizes is labour intensive and fatigue with transient loss in technologist concentration may result in errors, e.g. during manual pipetting. Staff should be trained to handle discrepant results between pool and individually tested constituents. Occasionally, a pool may be positive but subsequent individual testing of constituents are all negative. This could be due to contamination of the pooled sample, or non-optimal amplification conditions of the individual sample.

The laboratory team should be aware of the anticipated turnaround time for community samples to handle potential queries.

The laboratory director should continue to review prevalence periodically. Laboratories should be sufficiently nimble to revert to individual sample testing in anticipation of increase in community prevalence. The Dorfman Protocol is not efficient at high prevalence. At a prevalence of 30%, the laboratory will expend more reagents (Table 1).

The laboratory manager should monitor reagent consumption. While group testing at Level 2 (multiple donor transport media are pooled before nucleic acid purification) may save nucleic acid purification kits and PCR master mix reagents, the consumption of swabs remain the same and there may not be any savings in consumables such as pipettes tips, microtubes and transport media.

**New Developments**
Of note there is a recent interest towards a one-stage non-adaptive pooling strategy using compressed sensing.[67,68] In brief, compressed sensing enables the reconstruction of an original signal vector (**x**), from the pooled signals vector (**y**) in a sparse environment, by using an appropriately designed pooling matrix (**A**). In the context of SARS-CoV-2 PCR testing, **x** represents the individual samples results. Consider a n-dimensional vector which represents the results of many individual samples where n is large.

$$\mathbf{x} = \begin{pmatrix} x1 \\ x2 \\ \vdots \\ . \\ . \\ xn \end{pmatrix} \in \mathbf{R}^{\mathbf{n}} \text{ of real numbers}$$

*x1, x2, x3 ... xn* represent the results of 1st, 2nd, 3rd and nth individual sample respectively.

A laboratory may add various combinations of individual samples from **x** to make m pools, using a carefully designed matrix (**A**), where **A** is a matrix of size m by n. The result obtained for each of the m pools, is represented by a m-dimensional vector (**y**), where $\mathbf{y} = \mathbf{Ax}$. The aim is to reconstruct **x** based on **y**. As the number of pools (m) is less than the number of samples (n), this system of linear equations is underdetermined. In classical linear algebra, there is no unique solution.[69] However, if the vector is sufficiently sparse (low prevalence) and the matrix satisfies certain properties, we can reconstruct **x** based on **y** by finding the minimum sum of the absolute values of the vector components ($\ell 1$ minimisation).[70-73] In practice, this will require computer software.

Compressed sensing may be designed to account for noise (e.g. dropped PCR pools, imprecision in liquid dispensing) or not to account for noise (e.g. assumes procedure works perfectly, no PCR amplification failure etc.).[74]

Shental *et al.* recently devised and implemented a pooling strategy, named Pooling-Based Efficient SARS-CoV-2 Testing (P-BEST), based on compressed sensing where 384 patient samples were each aspirated six times into different pools, making a total of 48 unique pools, with each pool containing 48

samples from distinct individuals [384 x 6 = 48 x 48].[67] Their pooling design incorporated Reed-Solomon error correction codes. Reed-Solomon codes are used to protect messages against random occurring errors by adding redundancy to the information.[75,76] In P-BEST, each sample is aspirated six times into different pools for redundancy. As such, P-BEST is relatively robust for noise caused by failure of amplification in the pools, liquid dispensing imprecision or RNA variation. A requirement to implement the protocol is availability of a robotic liquid handler due to complex pipetting steps. At the prevalence of 1%, this implementation will be able to test up to eight times as many individuals with the same number of reagents. However, if prevalence increases beyond 1% then false positives will occur. Experimentally, they returned 1 false positive for 5 true positives at prevalence of 1.3% (5 true positives out of 384 samples). Interested readers may refer to Github Repository files that Shental *et al.* provided for an approach to implementation for compressed sensing with Reed-Solomon error correction codes or approach their electrical engineering or computer science department colleagues for collaboration.[77]

Ghosh *et al.* in their preprint described a one-stage non-adaptive technique which includes the use of noise compressed sensing techniques.[68] They developed variable pooling matrices suitable for 40, 70, 105, 195, 399, 961 and 1140 samples that involves lesser tests at selected prevalence.[78] Their protocol, Tapestry Pooling, is able to recover the quantitative viral loads. To aid laboratories through the pipetting steps to perform the combinatorial pooling, an Android application is available.

Compressed sensing can recover the underlying individual numerical value (e.g. viral load, CT value, concentration) of the individual samples and has been implemented by Ghosh *et al.*[79,80] Further developments on compressed sensing with the recovery of quantitative value of individual samples may expand group testing to other quantitative analytes in a one-stage non-adaptive approach.

## Conclusion

In conclusion, many analytes, especially in the field of infectious diseases, have the potential for pooled testing. Proper selection of analyte, ensuring sufficient sensitivity, monitoring prevalence, establishing a firm need for resource conservation, validation of assay method for pooling, configuration of the LIS and staff training are key to a successful and expectant implementation of group testing in the clinical laboratory. Pooling is not a panacea to overcome reagents shortage but may allow broader access to testing at the cost of reduced sensitivity. As the maxim dictates 'no test is better than a bad test', expansion in testing capacity by group testing should be carefully weighed against the implications of false negatives, and reduction in sensitivity must be mitigated. In addition to the Dorfman protocol, one-stage non-adaptive approaches using compressed sensing with error correction codes may be of interest to clinical laboratories with the potential to reduce false positives and negatives in the stipulated prevalence band.

**Acknowledgements:** The authors would like to acknowledge Prof Peter C. Iwen, Asst Prof Baha Abdalhamid (Nebraska Public Health Laboratory, US) and Prof Christopher R. Bilder (University of Nebraska-Lincoln, US) for assistance with group testing protocol in the early phase of the pandemic. We would also like to thank A/Prof Anders Kallner (Karolinska Institute, Sweden) for kindly sharing with us the historical perspective on group testing for TSH.

**Competing Interests:** None declared.

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